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Urinary Biomarkers of Tuberculosis: Potential for Diagnosis and Beyond

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Tuberculosis (TB) is the leading infectious cause of death worldwide. A major barrier to controlling the spread of this infection is the lack of reliable clinical tests that can be used for rapid diagnosis. Additionally, there is no good way to know if someone is responding to treatment. Biological markers were identified in urine that are capable of diagnosing pulmonary TB. This proposal aims to study these markers in groups of people suffering from active TB infection and compare them to urine from people who are exposed and at risk for developing disease. In addition, people who are being treated with antibiotics will be studied, to evaluate if these markers can be used to monitor whether treatment is working. If successful, these markers could dramatically improve the diagnosis of TB and help decrease the spread of drug resistant strains through early detection of treatment failures.
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1. Introduction

Tuberculosis (TB) remains the leading cause of deaths due to an infectious disease. A major barrier to control of the pandemic is the lack of rapid, point-of-care biomarkers. Urinary biomarkers were identified that can both diagnose active TB and decrease or increase, over time, while on anti-TB treatment. The objective of this proposal was to determine the reliability of these urinary biomarkers, both for diagnosis and assessment of treatment of TB. Aim 1 of this proposal aimed to prospectively follow urinary biomarker concentrations in participants during treatment. The working hypothesis was that urinary biomarker concentration will change over time and track with treatment response and mycobacterial load. Aim 2 was to determine whether urinary biomarker concentrations can differentiate between active TB, latent TB infection (LTBI) and un-infected healthy controls. The working hypothesis was that urinary biomarker concentrations will be increased in cases of LTBI, defined as having a positive interferon-gamma release essay (IGRA), as compared to healthy controls, but decreased when compared to cases of active TB.

2. Keywords

*Tuberculosis, biomarker, urine, treatment response*

3. Accomplishments

- What were the major goals of the project?

  The major goal of this project was to validate 10 urine metabolites as diagnostic for active tuberculosis and as potential markers of tuberculosis treatment response. Aims were as follows:

  o **Aim 1:** Prospectively follow urinary biomarker concentrations in participants during treatment to determine the relationship of the biomarkers to treatment response, bacterial burden and immunologic status.

    The *working hypothesis* is that urinary biomarker concentration will decrease over time and will be associated with bacterial burden as measured by sputum acid-fast bacilli (AFB).

  o **Aim 2:** Determine whether urinary biomarker concentrations can differentiate between cases of active TB infection, latent TB infection (LTBI) and un-infected healthy controls.

    The *working hypothesis* is that urinary biomarker concentrations will be increased in cases of LTBI, defined as having a positive interferon-gamma release essay (IGRA), as compared to healthy controls, but decreased when compared to cases of active TB.

To date we have completed mass spectrometry and statistical analysis on all participant urine samples and have prepared and submitted a scientific manuscript describing our findings from Aim 1. We are preparing a scientific manuscript describing our findings from Aim 2 and hope to submit for publication this year.
What was accomplished under these goals?

The following is unpublished data.

For Aim 1 the specific objectives were to quantify urinary metabolite levels in participant who were cured of TB to identify potential markers of treatment response. To date, we have completed experiments and analysis for longitudinal urine samples from 37 participants taken at the time of diagnosis and at week 2, 4, 8, 17, 26 and 52 after initiation of anti-tuberculosis therapy. Samples were grouped by participant and then randomized. Each urine sample was normalized to an osmolality of 150 mOsm and mixed with methanol 0.2% formic acid in a 1:1 mixture and then analyzed using an Agilent 6230 TOF LC/MS (Liquid chromatography–mass spectrometry), and Profinder B08 and Qualitative Analysis bio-informatic pipeline. All 10 metabolites tested showed a significant decrease during the course of TB treatment (figure 1). Kynurenine and diacetylspermine are significantly decreased at 2 weeks after the start of treatment. Each metabolite was significantly correlated with sputum AFB microscopy score (figure 2) and some metabolites were significantly higher in participants with high initial sputum AFB score when compared to low AFB score (figure 3). All metabolite abundances were normalized to creatinine concentrations and adjusted for age sex and participant weight.

These findings suggest that several urinary metabolites can be useful as prognostic markers of tuberculosis treatment response, even as early as 2 weeks after the start of treatment. As many of these metabolites are known inflammatory intermediates, these findings need to be validated in a cohort of treatment responders and non-responders to confirm their ability to identify those at risk for treatment failure.

![Figure 1: Molecule abundance decreases over course of treatment in clinically cured TB patients. Mean log2 abundance of 10 distinct urinary molecules over the course of TB treatment. All original LC/MS molecule abundances were normalized to urinary creatinine levels. Error bars represent 95% CI.](image-url)
The following is unpublished data.

Figure 2: Urinary abundance of molecules positively correlates with sputum mycobacterial burden. Scatterplots showing molecule abundances of each urine sample against its corresponding sputum AFB microscopy score. Data from all patients and at all time points are represented, with adjustment for age, sex, weight and intra-subject correlation. Each number represents a single participant. p value <0.01 for all molecules.

Figure 3: Mean molecule abundance is higher in urine of TB patients with high initial sputum mycobacterial load. Participants were separated by sputum AFB smear score at time of diagnosis (week 0). Initial AFB score of 3+ or 4+ were categorized as “high sputum load”; initial AFB score of 2+ or lower were categorized as “low sputum load”. Error bars represent 95% CI, log scale. All samples normalized to creatinine and adjusted for age, sex and weight.
The following is unpublished data.

The goals for Aim 2 are to study urinary metabolite levels in 100 participants with active TB, 100 participants with latent TB infection and 100 uninfected controls. To date, all the samples are prepped and 100 randomized samples have been run on the Agilent 6230 TOF LC/MS as described above. Preliminary analysis of this data set suggests that several of these metabolites may be increased in participants with HIV-TB co-infection.

Figure 4: Mean molecule abundance of metabolites differ in HIV infected individuals with active TB. Scatter plot showing molecule abundance in individuals who have active TB and HIV coinfection, active TB without HIV coinfection, latent TB (IGRA+) and IGRA negative controls. All samples normalized to creatinine and adjusted for age, sex and weight.

All urine samples have been run and metabolite abundance analysis is ongoing. We expect to validate these findings in the remaining urine samples. If validated these findings would give further evidence of a urinary biomarker signature as a potential diagnostic for active TB even in difficult to diagnose populations, such as HIV co-infection.
• What opportunities for training and professional development has the project provided?

Nothing to Report.

• How were the results disseminated to communities of interest?

We planned to disseminate the findings to the community by 1) publishing in peer reviewed medical journals and 2) presenting the results at international conferences and meetings. To meet these objectives, we have already prepared and submitted a manuscript describing the longitudinal treatment response cohort. We will also prepare and submit a scientific manuscript describing biomarker levels from patients with active TB, latent TB and healthy controls. Additionally, we have been invited to speak about our findings at the international infectious diseases conference IDWeek 2019 and at the national Tuberculosis Research Unit Annual meeting at the NIH.

• What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. Impact

As a result of this project we have identified potential early markers of TB treatment response. The current WHO recommendations state that TB treatment failure can only be evaluated after continued sputum positivity at 2-3 months after starting treatment. During these crucial months, a person is on ineffective or partially effective treatment, all the while being exposed to drug toxicity, increasing their risk for developing drug resistance and continuing to spread disease. An early marker of treatment efficacy could dramatically alter how TB is treated by identifying treatment failures as early as 2 weeks after starting therapy. Early identification of treatment failures would not only help individual patients, but could also help identify drug resistance, and decrease overall infectivity and thus TB incidence.

In addition, the discovery of N1N12 diacetylspermine as a reliable marker of TB treatment response has led to mechanistic studies on this molecule and its pathway in the macrophage both with and without concurrent TB infection. A role of spermines in TB pathogenesis has not been previously described. Understanding spermines and their role in TB pathogenesis can help our understanding of the disease and possibly lead to new therapeutics, drug targets or biomarkers of disease.

• What was the impact on the development of the principal discipline(s) of the project?

The ten metabolites being tested are thought to be immune intermediates. In addition to their potential as diagnostic and prognostic biomarker of TB, these metabolites could highlight previously unknown mechanisms of disease. As these biomarkers were identified using untargeted metabolomics, several of them have not been previously identified as associated with TB or host immunity. Diacetylspermine, specifically has not been previously associated with TB immunopathogenesis. Further studying this molecule and its role in TB pathogenesis can help our understanding of the disease and possibly lead to new therapeutics, drug targets or biomarkers of disease.

• What was the impact on other disciplines?

Nothing to Report.

• What was the impact on technology transfer?

Nothing to Report.

• What was the impact on society beyond science and technology?

The aim of this project is to validate urine biomarkers of TB. If validated this would be one step closer to developing a urine point-of-care test for diagnosing TB or monitoring response to therapy. A urine point
of care test for TB would dramatically change how TB is diagnosed and/or treated around the world and especially in resource limited settings, where disease incidence is high, or in difficult to diagnose populations, such as children or extrapulmonary disease.

5. Changes/Problems

• Changes in approach and reasons for change.
  Nothing to report.

• Actual or anticipated problems or delays and actions or plans to resolve them.
  Nothing to report.

• Changes that had a significant impact on expenditures.
  Nothing to report. There have been no changes that significantly impact expenditures.

• Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
  Nothing to report. There have been no significant changes in the use or care of human subjects, vertebrate animals, biohazards or select agents.

• Significant changes in use or care of human subjects.
  Nothing to report.

• Significant changes in use or care of vertebrate animals.
  Nothing to report.

• Significant changes in use of biohazards and/or select agents.
  Nothing to report.

6. Products

• Publications, conference papers, and presentations
  o Journal publications.
    Submission to JCI insight, awaiting review
  o Books or other non-periodical, one-time publications.
    Nothing to report.
  o Other publications, conference papers, and presentations.
    Results from this work have been presented at the regional and national Tuberculosis Research Unit meetings and the international infectious disease conference IDWeek Oct 2019.

• Website(s) or other Internet site(s)
  Nothing to report. There are no internet sites that disseminate the results of this research.
• Technologies or techniques

  Nothing to report. There have been no technologies or techniques that resulted from the research activities to date.

• Inventions, patent applications, and/or licenses

  Nothing to report. No inventions, patent applications and/or licenses have resulted from this project.

• Other Products

  Data or Databases: The metabolomics data generated from the participant urine samples has contributed a urine metabolomics database within the Rhee/Isa lab.
7. Participants & Other Collaborating Organizations

- What individuals have worked on the project?

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<td>Qianjing aided in sample preparation and analysis.</td>
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- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
  Since the last report Dr. Isa has taken a position as Associate Medical Director at Regeneron Pharmaceuticals.

- What other organizations were involved as partners?
  Nothing to report.

8. Special Reporting Requirements

COLLABORATIVE AWARDS:

Not applicable.

QUAD CHARTS:

Not applicable.
9. Appendices
Urinary Biomarkers of Mycobacterial Load and Treatment Response in Pulmonary Tuberculosis

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The authors have declared that no conflict of interest exists.

Key Words: N⁰, N¹²-diacylspermine, pulmonary tuberculosis, treatment response, mass spectrometry, urine biomarker, prognostic biomarker
Abstract

Background: Control of the tuberculosis (TB) pandemic remains hindered, in part, by a lack of simple and accurate measures of treatment efficacy. Current gold standard techniques rely on sputum-based assays that are slow and challenging to implement. Previous work identified urinary $N\text{, }N'$-diacetylspermine (DiAcSpm), neopterin, hydroxykynurenine, $N$-acetylhexosamine, ureidopropionic acid, sialic acid, and $m/z$ 241.0903 as potential biomarkers of active pulmonary TB (ATB). Here, we evaluated their ability to serve as linked biomarkers of TB treatment response and mycobacterial load.

Methods: We analyzed urine samples prospectively collected from two cohorts with ATB: 37 participants from African countries treated with first line TB therapy (HRZE) and followed for one year, and 35 participants from Haiti treated with either HRZE or an experimental drug followed for the first 14 days. Blinded samples were analyzed by untargeted high performance liquid chromatography-coupled-time of flight-mass spectrometry.

Results: Urinary levels of all seven molecules exhibited significant decreases by week 26 of successful treatment ($p=0.01$-$p<0.0001$), with positive correlations to sputum mycobacterial load ($p<0.0001$). Urinary levels of DiAcSpm exhibited significant decreases in participants treated with HRZE as early as 14 days ($p<0.0001$), but were unchanged in participants receiving ineffective therapy ($p=0.14$).

Conclusion: Reductions in urinary DiAcSpm, neopterin, hydroxykynurenine, $N$-acetylhexosamine, ureidopropionic acid, sialic acid, and $m/z$ 241.0903 correlate with successful anti-TB treatment and sputum mycobacterial load. Levels of DiAcSpm exhibited reductions capable of differentiating treatment success from failure as early as two weeks after the initiation of effective chemotherapy, commending its further development as a potentially simple, non-invasive biomarker of treatment response and bacterial load.
Funding: This work was supported by the Clinical and Translational Science Center at Weill Cornell College of Medicine (NIH/NCATS 1 UL1 TR002384-02 and KL2TR000458), the Department of Defense (PR170782), the NIH T32 training grant (T32-2T32AI007613-16), the National Institute of Allergy and Infectious Disease grants (NIAID K24 AI098627 and K23 AI131913), the NIH Fogarty International Center grants (R24 TW007988 and TW010062), the Abby and Howard P. Milstein Program in Chemical Biology and Translational Medicine, and the Tuberculosis Research Units Networks (TBRU-N, AI111143).
Introduction

Tuberculosis (TB) remains the leading cause of death worldwide due to a single infectious agent, and is the leading cause of death due to drug resistance (1). Control of the TB pandemic remains hindered, in part, by the limited range of clinically relevant diagnostic and treatment response biomarkers. Current gold standard methods for monitoring response to therapy continue to depend on sputum-based assays (2). While such methods are recognized for their ability to indicate disease severity and transmissibility, they are limited by practical inconsistencies in the ability to recover sputum, relative insensitivity of microscopy to bacterial burden and viability, and the inherently retrospective nature of culture-based methods which can often lag weeks to months behind the need for clinical decision making (3, 4). Recent advances in nucleic acid amplification-based methods such as GeneXpert® (Cepheid, Sunnyvale, USA) have begun to overcome some of these limitations. However, despite their increased speed and sensitivity, such tests continue to require sputum samples, are unable to distinguish live from dead mycobacteria, and remain prohibitively expensive to operate in low and middle-income countries, where more than 90% of TB cases occur (5, 6). These hurdles to the timely diagnosis of disease and verification of treatment efficacy are problematic because while awaiting test results, ineffective or only partially-effective treatments continue to promote clinical progression, continued transmission, and the emergence of drug resistance itself (7, 8).

Sputum mycobacterial load is a widely recognized marker of disease severity, correlating with clinical symptoms, the presence of cavitary lung lesions, and transmission rates (9–11). Novel biomarkers of TB diagnosis and treatment response would thus ideally enable rapid detection and quantification of sputum bacterial load, and should be inexpensive, simple, non-invasive, and non-sputum based. These ideal qualities would increase the ability to reach lower-resource
healthcare systems and reduce cost of diagnostic algorithms. In addition to their ability to
directly impact patient care, such biomarkers also represent a potentially powerful tool that could
accelerate the development of new TB drugs by providing quicker metrics of experimental drug
efficacy.

Urinary biomarkers have recently begun to emerge as clinically useful diagnostic markers of
infectious disease and prognostic markers of treatment efficacy (12–14). Urine is an easily
obtainable biological sample that is chemically complex, and contains breakdown products that
reflect changes in metabolism of host and pathogen (15). Moreover, growing evidence has
demonstrated that TB may elicit specific patterns of immune activation, including unique
transcriptional signatures and TB-specific T cell populations, some of which may be in the
metabolic profiles of the blood and urine of afflicted patients (16–21).

In previous work, we identified \( N_1, N_2 \)-diacetylspermine (DiAcSpm), hydroxykynurenine,
neopterin, \( N \)-acetylhexosamine, ureidopropionic acid, sialic acid, and an uncharacterized
molecule with mass-to-charge ratio \( m/z \) of 241.0903 as potential urinary biomarkers of active
pulmonary tuberculosis (ATB) (Table 1) (22). Levels of these urinary molecules decreased after
60 days of anti-TB treatment in 20 participants, supporting their utility for indicating active TB
disease (22).

In this report, we set out to investigate the potential of these same urinary metabolites to also
serve as biomarkers of TB treatment response and mycobacterial load.
Results

Levels of Urinary Molecules Decrease during TB Treatment

We first characterized the urine metabolic profiles of 34 participants treated for ATB using a blinded set of prospectively collected longitudinal urine samples from the REMoxTB trial (23) obtained through the Consortium for TB Biomarkers (CTB2). All participants received either 8 weeks of isoniazid, rifampin, pyrazinamide, and ethambutol (HRZE), followed by 18 weeks of isoniazid and rifampin, or received moxifloxacin in combination with isoniazid or ethambutol as detailed in the study description (23). Available participant characteristics are listed in Table 2.

All participants tested positive for sputum culture, AFB smear, or GeneXpert assay at the time of enrollment. All participants had negative AFB smears and sputum cultures at treatment termination (26 weeks) (Table S1). Participant urine samples were obtained prior to initiation of treatment (week 0) and at weeks 2, 4, 8, 17, 26 and 52 of the study. Samples were blinded, randomized and normalized by dilution to 150 milliosmoles (mOsm) prior to untargeted metabolomic profiling by high performance liquid chromatography-coupled high resolution time-of-flight mass spectrometry (HPLC/MS).

Urinary levels of DiAcSpm, hydroxykynurenine, neopterin, N-acetylhexosamine, ureidopropionic acid, sialic acid, and m/z 241.0903 all exhibited significant decreases by the end of treatment at 26 weeks after adjustment for age, sex, and BMI (linear mixed model p=0.01 - p<0.0001) (Figure 1). Of these, levels of DiAcSpm and hydroxykynurenine exhibited significant decreases after only 2 weeks of TB treatment (p<0.0001, p<0.0001, respectively) (Figure 1A, 1B) while levels of neopterin, N-acetylhexosamine, ureidopropionic acid, sialic acid, and m/z 241.0903 exhibited significant decreases from baseline between 4 and 26 weeks of treatment (p=0.01-p<0.0001) (Figures 1C-1G).
We next investigated whether urinary levels of any of these seven molecules correlated with sputum mycobacterial burden. Abundance data from all CTB2 urinary samples with their corresponding sputum AFB scores are presented on scatter plots, regardless of participant identification or treatment time point, in Figure 2. Regression slopes accounting for intra-subject correlation identified strong positive correlations between sputum AFB score and molecule abundance for all seven molecules (all p<0.0001).

To further investigate the relationship between the levels of these molecules and treatment-induced declines in sputum bacterial load, we assigned each of the 34 CTB2 cases into “high” or “low” mycobacterial burden groups based on sputum AFB scores at the time of diagnosis (week 0). Participants with initial AFB scores of 3+ or 4+ were categorized as “high sputum load” (n=11), and participants with initial AFB scores of 0, scanty, 1+ or 2+ were categorized as “low sputum load” (n=23) (Table S1). At baseline, levels of N-acetylhexosamine, sialic acid, and m/z 241.0903 differed significantly between high and low sputum groups (Figure 3A-C). In all cases, higher mycobacterial burden was associated with higher urinary molecule abundance. In contrast, by the time of treatment termination at week 26, levels of all molecules from both “high” and “low” initial sputum load groups converged to similar levels, consistent with their shared clinical endpoint of cure. The molecule DiAcSpm exhibited a similar trend as the three previously highlighted molecules, although the difference at baseline did not reach statistical significance (Figure 3D).
To independently validate our findings, we obtained and analyzed urine samples from a second cohort of 35 participants with ATB enrolled in an early bactericidal activity (EBA) study at the GHESKIO Centers in Port-au-Prince, Haiti. The study was designed to determine if the \textit{in vitro} activity of the FDA-approved anti-parasitic agent nitazoxanide (NTZ) against \textit{Mycobacterium tuberculosis} (Mtb) could serve as a mycobactericidal agent for drug-sensitive pulmonary TB (Walsh et al. 2019 pending publication?). Participants were recruited at the GHESKIO Centers and allocated into two treatment arms: 19 participants received NTZ for 14 days, while 16 participants received standard TB treatment of HRZE. Participant demographics are shown in Table 3. Inclusion criteria included either a sputum smear AFB score of at least 2+, or GeneXpert® MTB/RIF positivity for MTB at the medium or high level at the time of enrollment. Urine samples were collected before treatment on day 0, and on days 2, 4, and 14 after treatment initiation. All urine samples were blinded, randomized and normalized to 150 mOsm prior to untargeted metabolomic analysis by HPLC/MS. Additionally, overnight sputum samples were collected from each patient upon diagnosis, and continuously collected every two days for 14 days to monitor for changes in culture time to positivity (TTP) as a measure of treatment efficacy (Walsh 2019).

TTP data were mathematically converted to colony forming unit (CFU) values in this study to represent microbiologic data (24). Treatment with NTZ yielded no change in sputum culture CFU after 14 days, whereas treatment with HRZE resulted in the expected decrease in sputum culture CFU (Figure 4A). This finding suggests that NTZ did not have anti-mycobacterial activity during the trial. Urine samples corresponding to the start and end of treatment from each arm of this study thus enabled us to evaluate the performance of our urinary biomarkers in relation to treatment efficacy.
Linear mixed modeling demonstrated that in this GHESKIO cohort, HPLC/MS-measured mean urinary DiAcSpm decreased significantly in the HRZE arm (p<0.0001), but not in the NTZ arm (p=0.14), and reached statistical significance at the study endpoint of treatment day 14 (p<0.0001) (Figure 4B). This trend was also observed on the individual patient level when comparing changes in urinary DiAcSpm between days 1 and 14 (Figure 4C). Moreover, these reductions could be detected using an analytically independent, and commercially available, monoclonal antibody-based ELISA developed for clinical use (25) (Figure 5A and B).

Levels of urinary hydroxykynurenine, N-acetylhexosamine, ureidopropionic acid, and m/z 241.0903 showed similar significant decreases in mean abundance in participants treated with HRZE over the first two weeks, but did not achieve statistical significance when comparing the two treatment arms by the day 14 endpoint (Figure S1). Linear mixed modeling of maximum daily axillary temperatures taken on corresponding treatment days (0, 2, 4, 14) similarly failed to demonstrate significant difference between treatment arms (Figure S2).

Given the early response of DiAcSpm to effective therapy, we further investigated the association between DiAcSpm and mycobacterial burden in this cohort. To do so, we plotted baseline (day 0) calculated culture CFUs against corresponding urinary levels of DiAcSpm. As shown in Figure 6, increasing DiAcSpm concentrations correlated with higher mycobacterial burden in both HPLC/MS and ELISA results, as indicated by higher corresponding CFU values (p=0.0001 & 0.0003, r=0.3812 & 0.3318 respectively). This association was further validated using urine samples from a third cohort obtained from a study by Dupnik et al. (16) (Figure S3).
Current methods to objectively monitor TB treatment response and disease burden remain rooted in sputum-based assays that are prohibitively slow, complex, and often incompatible with the health care settings in which TB is most frequently seen. Clinicians must therefore rely on more subjective measures of symptom resolution while waiting several weeks or months for confirmation by sputum AFB and culture. Fast, affordable, and sensitive point-of-care tests thus constitute a major area of unmet medical need that is critical for control of TB at both the individual and population levels (26). Biomarkers from human biofluids are useful reporters of chemical and metabolic responses in different pathological states, but biomarkers of TB treatment response and disease burden remain conspicuously underexplored.

**Urinary biomarkers decrease with treatment and correlate with mycobacterial load**

Our study demonstrates that urinary levels of DiAcSpm, hydroxykynurenine, neopterin, N-acetylhexasamine, ureidopropionic acid, sialic acid, and m/z 241.0903 significantly decrease over the six-month course of treatment in 34 successfully treated TB cases. Previous work described elevated levels of these seven molecules in the urine of ATB cases when compared to non-TB pulmonary disease with an overall sensitivity and specificity of over 80% (22). Of these, kynurenine, neopterin, and sialic acid have all been previously reported to be increased in various human biofluids of ATB (27–31).

The rate at which these seven molecules decreased over 26 weeks of treatment varied. Some dropped precipitously within the first two weeks, while others showed gradual downward trends over the course of treatment. However, all were associated with significant reductions by the end of treatment, with some as large as eight-fold. These preliminary data thus suggest a potential
role for one or a combination of these seven urinary molecules to be developed into surrogate biomarkers of TB treatment response. Molecules exhibiting early declines could have a role in determining suitability of the medical regimen, while molecules with slower kinetics could serve as long-term monitoring tools to ensure continued drug effectiveness.

We found that urinary levels of all seven target molecules were also positively associated with sputum mycobacterial load. Urinary levels of N-acetylhexosamine, sialic acid, and m/z 241.0903 were initially significantly higher in participants with high sputum AFB scores at diagnosis, but eventually converged with levels from participants with low sputum AFB by 26 weeks.

Acetylated sugars such as N-acetylhexosamine are known components of the *Mtb* cell wall, and sialic acids are often expressed by pathogens in order to enhance intracellular survival and reduce host immune response (32). It is thus possible that these molecules may reflect specific *Mtb*-derived products. Biological origin notwithstanding, changes in mycobacterial burden may be the best indicator of treatment outcome for TB, and are routinely used in clinical practice to document treatment response (33). Correlative data between urinary molecule levels and corresponding sputum TB load presented in this study show promise for these urinary compounds to serve as surrogate markers of *Mtb* bacillary load.

**DiAcSpm’s potential role as a marker of antimycobacterial activity**

Polyamines, including spermine, spermidine, and putrescine, are present in all living organisms. They play important roles in major cellular processes such as growth and proliferation, and normally have tightly regulated intracellular levels (34). Current understanding of the polyamine pathway is shown in Figure 7, which illustrates that spermine can undergo catabolism either through direct oxidation by spermine oxidase (SMOX), or acetylation by spermidine/spermine
$N$-acetyltransferase (SSAT). It is postulated that $N$-acetylspermine can undergo a second acetylation step via SSAT, forming DiAcSpm. Acetylated polyamines are then exported from the cell via an ATP-dependent polyamine transporter (34).

In addition to DiAcSpm, several other metabolites in the polyamine catabolic pathway have previously been reported to be increased in TB states, including $N$-acetylisoputreanine (35). Considerable evidence have implicated polyamines in the pathogenesis of various bacteria. Several bacteria have been shown to upregulate polyamine catabolism in infected host tissues, and it has been suggested that acetylated end products of polyamine catabolism facilitate cellular export (34, 36). Macrophages have conversely been implied as a source of DiAcSpm. A study by Hamaoki and Nagata revealed that peritoneal macrophages from lymphoid tumor-bearing mice produced DiAcSpm in the presence of exogenous spermine (37). Most interestingly, an in vitro study from the 1950s demonstrated that exogenous spermine exhibited antimycobacterial properties after an unidentified enzymatic alteration (38). However, very little is known about the biological role of polyamines in TB immunopathogenesis (39, 40).

Previous work from this and other groups showed elevated levels of urinary DiAcSpm in ATB cases (22, 41). Our study now demonstrates that levels of DiAcSpm also decrease rapidly with effective TB treatment. This could indicate that polyamine catabolism increases in TB infected lung tissues and subsequently decreases with resolution of the infection. An alternative explanation could involve an increased conversion of spermine to DiAcSpm by macrophages during active infection, in an attempt to produce antimycobacterial effects similar to those observed by Hirsch et al. in vitro (38). In this scenario, levels of DiAcSpm would decrease with treatment since macrophages would face decreasing Mtb bacterial burden as the infection clears.
Interestingly, our study demonstrates that levels of DiAcSpm may be sufficiently sensitive to
differentiate treatment success from failure during the first 14 days of anti-mycobacterial
therapy. This suggests a potentially important role of DiAcSpm in facilitating EBA drug trials,
which currently rely on a time-consuming method of counting amounts of viable CFUs from
sputum cultures.

From a translational perspective, DiAcSpm is a regular constituent of human urine, consistently
accounting for 0.5% of total excreted urinary polyamines (42). DiAcSpm is not reabsorbed by
the glomerular filtration system, and there is minimal diurnal variation in its urinary content
among healthy individuals, suggesting tight control of its secretion (42, 43). These remarkable
qualities thus commend further investigation of urinary DiAcSpm as a candidate biomarker of
treatment response.

**Conclusion**

We have identified several candidate prognostic biomarkers of TB treatment response. Levels of
urinary DiAcSpm specifically show early and significant decrease in cases of successful TB
treatment, suggesting its potential for development into an early biomarker of TB treatment
efficacy.
Methods

Study Design

Longitudinal urine samples from 34 participants successfully treated for ATB were obtained from the CTB2 biorepository for urinary metabolite analysis over the course of treatment. Sputum mycobacterial data were made available in order to study correlation between urinary metabolites and \textit{Mtb} burden. Additional urine samples were obtained from the GHESKIO Centers in Port-au-Prince, Haiti from 35 participants enrolled in an EBA trial, and were used for urinary metabolite analysis in cases of ineffective treatment (Walsh 2019).

CTB2 Longitudinal Cohort

The CTB2, comprised of the Global Alliance for TB Drug Development, the TB Trials Consortium, and the AIDS Clinical Trials group, has created a collaborative biobank in order to accelerate biomarker discovery and validation for the diagnosis and treatment of TB. In collaboration with CTB2, we obtained prospectively collected longitudinal urine samples from 34 participants treated for confirmed ATB (Table 2). By request, clinical information for these samples were blinded to us until completion of metabolite analysis. Participants were recruited for two separate studies in unspecified African countries and followed over one year. Treatment consisted of either 8 weeks of rifampin, isoniazid, pyrazinamide, and ethambutol (HRZE), followed by 18 weeks of isoniazid and rifampin, or was replaced in part by moxifloxacin as outlined in the REMox Trial (23). Information on the specific treatment regimen corresponding to each participant, medication compliance, and drug susceptibility was not provided to us. Urine samples from each participant were collected at baseline (week 0), and at weeks 2, 4, 8, 17, 26, and 52 post-treatment. Sputum culture and AFB data were obtained at weeks 0, 4, 8, 26, and 52.
post-treatment (Table S1). Chest X-rays (CXRs) and GeneXpert data were recorded at the time of diagnosis for 33 and 28 patients, respectively. All participants had either sputum AFB, culture, or GeneXpert positivity at time of diagnosis. All patients showed no culture or AFB positivity at treatment termination (26 weeks).

Urine sample collection, storage, and shipment (CTB2)
Clean-catch urine samples were stored at -80°C in the Fischer BioServices facility in Bishop’s Strotford, England. Samples were shipped via PDP Couriers on dry ice, with constant temperature monitoring using a United Technologies Sensitech TempTale4 system, to the Belfer Research Labs at Weill Cornell Medicine and stored at -80°C until time of analysis.

Assignment to sputum mycobacterial load group (CTB2)
AFB seen under smear microscopy are classified as 4+, 3+, 2+, 1+, scanty, or 0, with greater numbers denoting higher bacillary loads. To create a dichotomous variable for mycobacterial load, the 34 CTB2 cases were assigned into “high” or “low” mycobacterial burden groups based on sputum AFB scores at the time of diagnosis (week 0). Participants with initial AFB scores of 3+ or 4+ were categorized as “high sputum load” (n=11), and participants with initial AFB scores of 2+, 1+, scanty, or 0 were categorized as “low sputum load” (n=23).

GHESKIO Cohort
Urine samples were collected from 35 participants with confirmed drug-sensitive ATB at the GHESKIO Centers as part of a 14-Day EBA study of nitazoxanide (NTZ) for the treatment of pulmonary TB (Table 3). Urine was collected from each participant pre-treatment on day 0, and
on days 2, 4, and 14 of treatment. Participants were allocated into two treatment groups: 19 participants were treated with a 14-day course of NTZ, and 16 participants were treated with the standard HRZE therapy as defined by the WHO. Of the 16 control HRZE participants included in our analysis, 10 were randomized control participants from the clinical trial, and 6 were additional control participants enrolled in a pilot phase of the trial to validate laboratory assays. Overnight sputum samples from this cohort were collected every two days and cultured using the Mycobacterial Growth Indicator Tube (MGIT) automated liquid culture system (BACTEC; BD, Franklin Lakes, NJ) to generate time to positivity (TTP) data (Walsh 2019). TTP data was subsequently mathematically converted to CFU values in this study, using the formula derived by Diacon et al (24).

Urine sample collection, storage, and shipment (GHESKIO)
Clean-catch urine samples were collected in sterile cups and immediately refrigerated at -4°C for 1-7 hours. Urine was then aliquoted on ice and stored at -80°C in GHESKIO facilities in Port-au-Prince, Haiti, until shipment to NYC. Shipments were sent on dry ice via World Courier from GHESKIO to the Weill Cornell Center for Global Health laboratory in New York, and stored at -80°C until time of analysis.

Sample Preparation
Samples from all cohorts were stored in a -80°C freezer at the Belfer Research Building at Weill Cornell Medicine until testing. Samples were blinded, randomized and prepared in sets of 20 to 25. The osmolality of each sample was measured using an Advanced Instruments model 3250 Micro-Osmometer. Samples were then centrifuged for 10 minutes at 10,000rpm in PALL nanosept centrifuge devices. Filtered substrate was diluted with MilliQ water to 150mOsm in
order to standardize the salt concentration within each sample. All samples below 150mOsm prior to dilution were excluded from analysis. Diluted samples were mixed with LC/MS grade methanol containing 0.2% formic acid at a 1:1 sample to solvent ratio for HPLC/MS analysis. Each set of 20 to 25 samples was run with a standard solution that consisted of 10µM of glutamate, succinate, lysine and nicotinic acid to ensure adequate sensitivity of the LC/MS. Pooled urine samples were included periodically throughout each set to allow for normalization of peak intensities and monitoring of mass spectrometer sensitivity within each run. A third of the total urine samples from both CTB2 and GHESKIO cohorts were randomly selected for replicate runs to ensure data reproducibility. Replicate runs were performed using previously un-thawed urine aliquots, independently randomized, and run in sets of 20 to 25 samples with the above standard solutions.

HPLC/MS Analysis:

Samples were analyzed using an Agilent Technologies 6230 TOF LC/MS. Liquid chromatography (LC) separation was achieved using a Cogent 4 Diamond Hydride column with an initial gradient of 85% LC/MS grade acetonitrile containing 0.2% formic acid, followed by a gradual increase in hydrophilicity to 95% LC/MS grade water containing 0.2% formic acid. Detected ions were indexed and characterized using their ion mass to charge ratio (m/z) and chromatographic retention time. Data was analyzed using Agilent Technologies Qualitative Analysis B.07, Agilent Technologies MassHunter Profinder B.08, and the XCMS software. Compound identification was achieved using known m/z and retention time coupled to chemical standards of targeted compounds run with each set of urine samples. Identity of DiAcSpm was further confirmed using MS/MS fragmentation analyses of chemical standards and random
patient urine samples. DiAcSpm chemical standards at five known concentrations (50nM, 100nM, 500nM, 1µM, and 5µM) were included within each run to create standard curves for urinary DiAcSpm concentration calculation.

Urinary DiAcSpm ELISA kits

Previously unthawed urinary samples were used for this portion of the analysis. 50µL of vortexed urine was centrifuged for 5 minutes at 1500rpm. Urine was serially diluted 4 to 9 times accordingly in order for resulting concentrations to remain within ELISA kit detection range. Absorbance was measured using a Spectramax M2 microplate reader at 490nm. Each sample was measured in duplicates, and all measured DiAcSpm concentrations were within range of the standard curve. Final results were adjusted for initial dilution ratio, and further normalized to respective urinary creatinine concentrations, with a final unit of nmol/g creatinine.

Creatinine Normalization:

All molecule abundances were additionally normalized to creatinine concentrations of corresponding urine samples using a creatinine colorimetric assay kit (Sigma Aldrich Catalog number MAK080). Absorbance was measured using a Spectramax M2 microplate reader at 570nm. Each sample was measured in duplicates, and all measured creatinine concentrations were within range of the standard curve.

Statistical Methods

All normalized molecule abundances were log2 transformed for analysis and visualization. Data analysis was performed using STATA SE version 15.
Longitudinal Analysis

Longitudinal trends of target urinary molecules were fitted using a mixed model. We estimated the effects of treatment at each time point as fixed effects while incorporating subject-specific abundances as random effects in the model. Hypotheses of factor variables and their interactions were assessed using the Wald test provided by the STATA margins command.

CTB2 cohort: The effect of AFB on longitudinal abundance profiles was estimated using a binary variable (low vs high AFB) in the model. We included interaction terms between AFB and time in order to account for confounding trends over time due to AFB. Time and AFB effects were calculated while adjusting for BMI, age, and sex.

GHESKIO cohort: We included the two treatment arms of NTZ and HRZE as binary variables in our statistical model. Interactions between treatment and time were included and assessed. Treatment and time effects were calculated while adjusting for age and baseline weight.

Molecule abundance correlation with Sputum AFB Score (CTB2)

We fitted a linear regression of metabolite abundance against corresponding AFB scores and assessed their correlation. Data from all available CTB2 cohort samples and time points were included. We used the robust variance calculation method in order to account for intra-subject correlation.

GHESKIO cohort TTP and CFU

Effect of treatment (NTZ vs HRZE) was assessed by fitting a mixed model on longitudinal arrays of CFU data. CFU values were mathematically converted from clinically measured patient TTP data, using the formula $\log_{10}(\text{CFU}) = 16.41 - 5.17 \times \log_{10}(\text{TTP})$ derived by Diacon et al (24).
Graphical representation of the original TTP data was shown in a study by Walsh et al (Walsh 2019).

**Study Approval**

Consent was obtained from all participants by local health workers during meetings conducted in the local language. All participants provided written informed consent prior to inclusion in the clinical cohort studies. IRB approval was obtained for this present study at Weill Cornell Medicine. Studies from which CTB2 and GHESKIO samples were obtained have IRB approval at their respective institutions.
Author Contributions

F.I. and Q.X. designed and conducted the experiments, acquired and interpreted MS data, and evaluated urinary molecule performance as markers of successful TB treatment. M.H.L. performed statistical analyses. J.M.B., K.F.W., K.M., and K.M.D. collected and provided clinical samples and clinical data. Q.X., F.I., and K.Y.R. wrote the manuscript. D.F. and K.Y.R. supervised and coordinated the work. All authors reviewed the manuscript, agreed with the results, and provided insight.

Acknowledgements

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27. Fitzgerald BL et al. Elucidation of a novel human urine metabolite as a seryl-leucine glycopeptide and as a biomarker of effective anti-tuberculosis therapy [Internet]. *ACS Infect Dis* [published online ahead of print: December 26, 2018]; doi:10.1021/acsinfecdis.8b00241


Figure 1

Molecule abundance decreased over course of treatment in clinically cured TB patients (n=34). Mean log₂ fold change of each urinary molecule from baseline abundance at time of diagnosis (week 0). Red line represents no change from baseline. All original HPLC/MS molecule abundances were normalized to corresponding urinary creatinine levels. Error bars represent 95% CI. Statistical difference between adjacent time points was determined using the Wald test and represented by *. (*, p<0.05; **, p<0.01; ****, p<0.0001)
Figure 2 Urinary molecule abundance positively correlates with sputum mycobacterial burden. Scatterplots depict molecule abundances of each urine sample against its corresponding sputum AFB score (all p<0.0001). Vertical axes represents log₂ of LC/MS molecular abundances after creatinine normalization. Data from all CTB2 patients (n=34) and at all time points are represented, with adjustment for intra-subject correlation. Regression line is represented in red ($r^2 = 0.0928 – 0.2505$).
Figure 3 Mean molecule abundance is higher in urine of TB patients with high initial sputum mycobacterial load. Mean HPLC/MS abundance in log$_2$ scale separated by initial sputum mycobacterial load of sialic acid (A), N-Acetylhexosamine (B), m/z 241.0903 (C), and Diacetylspermine (D). Participants were separated by sputum AFB smear score at time of diagnosis (week 0). Initial AFB scores of 3+ or 4+ were categorized as “high sputum load” (n=11, in red); initial AFB scores of 2+ or lower were categorized as “low sputum load” (n=23, in blue). Error bars represent 95% CI.
Figure 4. Urinary $N^1$, $N^{12}$-diacetylspерmine levels differentially decrease in successfully treated patients within the first 14 days. (A) Sputum culture CFUs show no change in bacterial burden of TB patients treated with 14 days of NTZ (n=19, in red). CFUs decrease during treatment with rifampin, isoniazid, pyrazinamide, and ethambutol (HRZE) (n=16, in blue), demonstrating decreased bacterial burden. (B) HPLC/MS-measured urinary DiAcSpm decreases significantly in participants treated with HRZE (blue) but not in those treated with NTZ (red). Solid dots represent mean fold change from baseline levels in log$_2$ scale. Error bars represent 95% CI and do not overlap at day 14. Dotted red line represents no change from baseline. (C) HPLC/MS-measured urinary DiAcSpm levels of individual participants. Each line represents an individual participant. Dotted line represents no change from baseline. All values have been normalized to corresponding urinary creatinine concentration.
Figure 5. Change in urinary \( N^1, N^{12} \)-diacetylspermine levels confirmed using different modality of detection. (A) ELISA-measured urinary DiAcSpm levels demonstrate significant concentration decreases in participants treated with HRZE (n=16, in blue) over 14 days, but not in those treated with NTZ (n=19, in red). Solid dots represent mean fold change from baseline levels in \( \log_2 \) scale. Error bars represent 90% CI and do not overlap at day 14. (B) Changes in ELISA-measured urinary DiAcSpm levels of individual participants over 14 days. Each line represents a single participant. Dotted line in each graph represents no change from baseline. All values have been normalized to corresponding urinary creatinine concentration.
Figure 6. Urinary $N^1, N^{12}$-diacetylspermine concentration correlates with culture measures of mycobacterial burden. Scatterplots with regression lines show correlation between calculated CFU upon diagnosis (Day 0) and DiAcSpm concentration (n=35). Increasing DiAcSpm concentration is associated with an increase in CFU, which is in turn a microbiological measure of mycobacterial burden. DiAcSpm concentrations were determined using (A) LC/MS chemical standard abundances ($p = 0.0001$, $r^2 = 0.3812$) and (B) ELISA ($p = 0.0003$, $r^2 = 0.3318$). All values have been normalized to corresponding urinary creatinine concentrations.
Figure 7. Polyamine synthetic and catabolic pathway. Circled enzyme spermidine/spermine N′-acyltransferase (SSAT) is hypothesized to be responsible for the production of DiAcSpm through a second acetylation process. SMOX – spermine oxidase. Solid lines represent known pathways; dotted line represents postulated pathway.
Table 1. Characteristics of molecules analyzed in urinary samples of ATB cases

<table>
<thead>
<tr>
<th>Mass-to-Charge Ratio (m/z)</th>
<th>Retention Time (min)</th>
<th>Predicted Formula</th>
<th>Preliminary Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>133.0600</td>
<td>1.56</td>
<td>C&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Ureidopropionic acid</td>
</tr>
<tr>
<td>144.1241</td>
<td>14.92</td>
<td>C&lt;sub&gt;14&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sup&gt;1&lt;/sup&gt;, N&lt;sup&gt;12&lt;/sup&gt;-diacetylspermine</td>
</tr>
<tr>
<td>186.0762</td>
<td>2.08</td>
<td>C&lt;sub&gt;8&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;NO&lt;sub&gt;6&lt;/sub&gt;</td>
<td>N-Acetyllhexosamine</td>
</tr>
<tr>
<td>225.0845</td>
<td>6.22</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Hydroxykynurenine</td>
</tr>
<tr>
<td>241.0903</td>
<td>1.77</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>254.0859</td>
<td>3.32</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;11&lt;/sub&gt;N&lt;sub&gt;5&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Neopterin</td>
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<tr>
<td>310.1148</td>
<td>2.56</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;19&lt;/sub&gt;NO&lt;sub&gt;9&lt;/sub&gt;</td>
<td>Sialic acid</td>
</tr>
</tbody>
</table>

<sup>A</sup>All molecules were previously elucidated by Isa et al. 2018
Table 2. Clinical characteristics of participants from the CTB2 longitudinal cohort

<table>
<thead>
<tr>
<th>Participant Characteristics (n=34)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age, years (range)</td>
<td>33.9 (18-59)</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>21M 13F (38.2%)</td>
</tr>
<tr>
<td>Mean BMI (range)</td>
<td>18.7 (14.4-25.2)</td>
</tr>
<tr>
<td>HIV+ (%)</td>
<td>4 (11.8%)</td>
</tr>
<tr>
<td>GeneXpert + (%)</td>
<td>28 (100%) ^A</td>
</tr>
<tr>
<td>Cavitation on CXR (%)</td>
<td>31 (93.9%) ^B</td>
</tr>
<tr>
<td>Culture and AFB neg by 8wks (%)</td>
<td>19 (55.9%)</td>
</tr>
<tr>
<td>Culture and AFB neg by 26wks (%)</td>
<td>34 (100%)</td>
</tr>
</tbody>
</table>

^A 6 patients did not undergo GeneXpert testing at time of diagnosis

^B 1 patient did not undergo chest X-ray testing at time of diagnosis
### Table 3. Clinical characteristics of participants from the GHESKIO cohort

<table>
<thead>
<tr>
<th></th>
<th>Participants with ATB under Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HREZ Treatment (n=16)</td>
</tr>
<tr>
<td><strong>Mean Age, years (range)</strong></td>
<td>32.1 (18-52)</td>
</tr>
<tr>
<td><strong>Sex (% female)</strong></td>
<td>9M 7F (43.8%)</td>
</tr>
<tr>
<td><strong>Mean Weight, pounds (range)</strong></td>
<td>119.0 (101-137.2)</td>
</tr>
<tr>
<td><strong>HIV+ (%)</strong></td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>GeneXpert + (%)</strong></td>
<td>16 (100%)</td>
</tr>
<tr>
<td><strong>Cavitation on CXR (%)</strong></td>
<td>8 (50%)</td>
</tr>
</tbody>
</table>